EVALUATION OF CYTOTOXIC POTENTIAL OF *PETROSELINUM SATIVUM* AND *MAGNOLIA* LILLIFERA EXTRACTS ON HUMAN CANCER CELLS

Dissertation-II

BY

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DECLARATION

This is to clarify that the declaration statement made by the student is correct to the best of any knowledge and belief the project proposal based on the research work learnt is fit for the submission and partial fulfillment of the conditions for the award of M.Sc. (Hons.) Biotechnology from Lovely Professional University, Phagwara.

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CERTIFICATE

This is to certify that SANA SHAFI bearing registration no. 11501978 has completed dissertation project report, titled "**EVALUATION OF CYTOTOXIC POTENTIAL OF *PETROSELINUM SATIVUM* AND *MAGNOLIA LILLIFERA* EXTRACTS ON HUMAN CANCER CELLS"** under my guidance and supervision. No part of the report has ever been submitted for any other degree at any university.

This report is fit for submission and partial fulfillment for the award of M.Sc. (Hons.) Biotechnology.

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ABSTRACT

Cancer is fatal and life threatening disease, responsible for 5-6% of death annually. The number of cases has drastically increased. People living with cancer were found to be 32.6 million (within 5 years of diagnosis) in 2012 worldwide. By 2030, it has been estimated that by 2030 there will be 26 million new cases related to cancer and 17 million deaths due to cancer per year**.** Cancer cell divide more rapidly than normal cells, and chemotherapy effectively target those cells but healthy cells can be damaged too. The chemotherapy can harm cells in bone marrow, where blood is produced which may lead to several problems. So therefore the research for the drugs of natural origin as an alternative drug, which have fewer side effects and are less toxic and more powerful in their mechanism of action, is important line of research. The medicinal plants can be potential source for the treatment as well as cure for the cancer. *Petroselinum sativum* and *Magnolia lillifera* were used in the current study. The aim to evaluate cytotoxic potential of these extracts derived from these plants hence anti-cancer properties. Results from MTT assay showed that *Petroselinum* plant extract and *magnolia* plant extract inhibit the proliferation of human cancer cell lines (MCF-7 and MDA-MB-231) in a dose dependent manner. Further most this study revealed that *Magnolia* plant extract was showing the maximum cytotoxic effect and the underlying mechanism of inhibition of growth involved cell death by apoptosis. The overall study determined the potential application of *Petroselinum sativum* and *Magnolia lillifera* for treating human cancer cells.

CHAPTER 1

INTRODUCTION

Medicinal plants represent a vast potential resource for anticancer compounds. The importance of medicinal plants is in the possible access to complex molecular structures that would be challenging to synthesize in the laboratory. Various mechanisms may result from antitumor activity of medicinal-plant-derived compounds, including cytoskeletal proteins effects that have a key role in cell division, DNA topoisomerase enzymes inhibition, anti-protease or antioxidant activity, the immune system stimulation, etc. Worldwide Medicinal plants are continued to be subject to extensive screening, in an order to develop more effective anticancer treatments.

60% of the drugs that have been isolated from plants are currently used for testing of potential of treatment of cancer and the plant kingdom has been the most important source of such principles ever since. Medicinal plants contribute a common pathway for cancer treatment in many countries around the world. Presently, more than 3000 plants all over world have been reported to have anticancer properties. Worldwide, the incidence of plant-derived products is between 10 to 40 percent with this rate reaching 50 percent in Asiatic patients for the treatment of cancer.

Petroselinum sativum is commonly known as parsley and *Magnolia lillifera* is commonly known as *Magnolia*. The Researchers from Hungary confirmed that *parsely* contains antioxidants that have anti-carcinogenic, anti-inflammatory properties, boost liver health and relax smooth muscles. It also contains significant nutrients and numerous bioactive compounds such as *coumarins* and *flavonoid*s. *Apigenin*, a *flavonoid* has been proven to suppress tumor cells aswellas shown to significantly slow inflammation and neutralize free radicals. The ability of *Apigenin* to show anti-tumor properties lies in its potential to prevent tumor by inhibiting formation of blood vessels. *Apigenin* was also found to be helping in preventing the migration of bladder cancer cells which in turn inhibited tumor growth by blocking the action of *ME*K kinase 1. This was published by researcher from china's Jiangsu College of agriculture and forestry. *(Boldizsár et al., 2012)*

Another compound found in parsley is *coumarin* (but in lower amount). *Coumarins* are having anti-coagulating properties as they are proven to be the natural blood thinning agent. It has been

found to improve circulation in case of inflammation or edema. As stated earlier that parsley has ability to relax smooth muscles, which it does by actin polymerization. This property of parsley has vital importance for patient suffering from asthma, as over- contraction of muscles around the lungs take place in case of severe asthmatic attack. *(Liu Q et; al:2011*)

Another study by Turkey's Hacettepe University Faculty of Medicine found ability of parsley to encourage healing properties. They found that there were fewer complications in bone marrow stem cell transplantation in children after consumption of parsley in high amount. The study was conducted on 41 children who underwent hematopoietic stem cell transplantation in children by analyzing their diet. The analysis report revealed that there were improved results in the children who consumed parsley as compared to children who ate different diet other than parsley. (*Koksal*e. et al., 2002)

In another study, Researcher of University of Missouri found that flavonoids *c*ompound present in parsely such as apigenin, can stop multiplication of certain breast cancer cells. In this experiment and rats were developed with certain type of breast cancer and were exposed to apigenin. Those rats having exposure to apigenin were found to develop less or reduced tumor formation while as the rats which were not exposed to apigenin had higher rate of tumor formation in comparison. There was also significant delay in tumor formation in rats having apigenin exposure. This finding may have impact on the Hormone Replacement Therapies that is most likely to accelerate breast tumor development in women. It was found that use of certain synthetic hormones in Hormone Replacement Therapy accelerates the development of breast tumor. In this study, the rats were exposed to MPA-Medroxy progesterone also called as progestin, one of the chemicals used in Hormone replacement therapy, most commonly received in the United States -- the same synthetic hormone that accelerates breast tumor development. MPA was found to aid the formation of blood vessels within tumor cells to receive nutrients to grow and multiple. When these rats were exposed to *apigenin* it was found to block the formation of blood vessels thus reducing overall number of tumor. *(B. Mafuvadze, et al,.2011).*

The study was also carried out on the *Petroselinum sativum* to check the activity of seed extracts on the human hepato cellular carcinoma cells (*HepG2*). In this experimental study the *HepG2* cells were exposed to parsley seed extract for 24h. After this cell morphology using phase

contrast microscopy was studied, viability assays were also conducted. The results revealed that extract from *Petroselinum sativum* has notably reduced the viability of HepG2 cells. However, this study was conduct by using two extracts one from *Petroselinum sativum* and another from *Portula caoleracea. (Farshori NN et al., 2014).*

The aqueous extract from the aerial parts and roots of *Petroselinum sativum* proved to have the therapeutic effect on kidney calculi. The study was conducted on thirty-six male Westar rats which were divided into six groups (viz. A, B, C, D, E, F) and underwent treatment for 30 days. The normal control was group A and group B was given drinking water containing ethylene glycol. The group C, D, E and F were used as subjects for treatment and received 1% of ethylene glycol from the day zero. The group C and D rats were given aerial parts aqueous extract of 200 and 600 mg/kg body weight, respectively, and the rats in group E and F received aqueous extract of roots of 200 and 600 mg/kg body weight, in drinking water, respectively, starting from the 14^{th} day of the experimentation. It was seen on 14^{th} and 30^{th} day, the level of magnesium in serum decreased notably while as level of calcium in serum increased in group B as compared with the control group. In case of groups of C, D, E, and F, which were under treatment, the deposition number notably reduced as compared to group B on day 30^{th}. In group B the kidney weight was increased as compared to control group and kidney weight in groups under treatment significantly decreased. This study significantly proved the aqueous extract of the aerial parts of *Petroselinum sativum* reduces the number of calcium oxalate deposits and has therapeutic effect on calcium oxalate stones present in rats suffering from nephrolithiasis. *(Jafarsaeidi et al., 2012).*

Magnolia lillifera is another plant under study and has found be useful for cancer treatment. The experiment carried out on the *Magnolia* seed extract showed chemo preventive and cytotoxic effects on MDA-MB231 breast cancer cell line. The MTT assay was used to evaluate the cytotoxic effect of *Magnolia* seeds on non-tumor cells and human cancer cells, at various concentrations ranging from 1-200 µg/mL. The TUNEL assay was used to evaluate the apoptotic activity of *Magnolia* seeds (MDE) 25uL/ml on breast cancer cell MDA-MB231 and immune fluorescence analysis was used to detection of caspase-3 for 48 h each. The test subjects were athymic mice which were developed with MDA-MB231 cells. These mice were intra peritoneally injected with different dosage of MDE, (1 and 50 mg/kg) during 28 days and its

chemo preventive effect was evaluated. During this span of 28 days, weight and growth of tumor cells were also measured. The result revealed that MDE has cytotoxic effect on cancer cell MDA-MB231. DNA fragmentation in MD-MB231 also showed that it exerted pro-apoptotic activities. The results suggested that MDE (*Magnolia dealbata* seeds) may be the source of honokiol and Magnolol, which are bioactive compound.*(Alonso-castro AJ et al., 2014)*

Magnolol, derived from *Magnolia* has also been found to have potential to treat lung cancer. The study conduct on *Magnolol* revealed that it is having anticancer property, but it not clear yet whether the apoptosis of lung cancer cells can be induced by *Magnolol*. This study stated that *Magnolol* could reduce and inhibit the cell growth. It was also found that release of lactate dehydrogenase was increased, and in case of A549 carcinoma cell, *Magnolol* could modulate cell cycle. The study also suggested that capillary tube formation in case of umbilical vein endothelial cells of human was inhibited as well as fibroblast growth factor-induced proliferation was also inhibited by *Magnolol. (Seojuet et al., April 2011).*

The China and Japan has been using bark of *Magnolila officinal is* as traditional medicine to treat diabetes. The stem bark of *Magnolia* contains major bioactive constituents such as 4-o-*methyhonokiol (MH), Magnolol* (*MAG) and honokiol* (*HON*). It was revealed in the recent study that *HON* and *MAG* could relieve accumulation of fat and resistance to insulin in high-fat diet that was fed to mice. In addition to this it was also seen in type 2 diabetic models, MAG reduced fasting blood sugar and insulin level in plasma without causing a change in body weight gain or adipocytes glucose uptake. Another extract from *Magnolia officinalis* BL153, was reported to partially reduce the heart damage along with lipid accumulation, inflammation, apoptosis in case of high-fat diet-fed mice. (*ZhiguoZhang et al., 2015).*

The study was done for chemical analysis of essential oil found in *Magnolia liliflora* and its pharmacological effect in nursing pregnant women having decubitus ulcer. The essential oil composition of *Magnolia liliflora* were identified as: *eucalyptol, bicyclo-heptan-2-one, β-pinene,1,7,7-trimethyl-,(1S),a,a,4-trimethyl,3-cyclohexene-1-methanol,3-cylohexen-1-ol, camphene, alpha-pinene, 4-methyl-1-(1-methylethyl).* The composition of these oils wasanalyzed by GC-MS. In order to check the pharmacological effect of the essential oil of *M.liliflora* , serum IL-4, IF-γ and the area effected with decubitus ulcer in pregnant women, were used. It was

reported that serum IL-4 level was decreased and reduced decubitus ulcer. This result indicated that essential oil in *M.lilifloral*iseffective. (*Liang Zhenhong et al., 2011)*

CHAPTER 2

REVIEW OF LITERATURE

2.1 ANTICANCER DRUGS

2.1.1 TAXOL

Taxol, also called as *paclitaxel*, is obtained from bark of *Taxus brevifolia*, the *pacific yew tree*. It is natural anti-cancer drug and has been used in treating of ovarian cancer, breast cancer, and lung cancer and for treating Kaposi's sarcoma. In 1962 the U.S. Department of agriculture based researchers collected sample from bark of pacific yew for the first time and found that extract from bark possess the cytotoxic activity. In 1977 NCI (National institute of cancer) treated mouse melanoma B16 model with the extract from paclitaxel and reported the anti-tumor activity and thus it was selected for clinical development. (*Kreeger KY.et al., 2001).*

Paclitaxel, under brand name of Taxol, is usually given as intravenous injection. There is no tablet or pill form of Taxol. It can also cause allergic reaction and even can cause inflammation of veins, as it is an irritant. *(Smit Egbert F. et al., 2003.)*

Taxol works by reducing the ability of tumor cells to divide. It is given along with the chemotherapy medicines in combination and usually given after the patient is under gone surgery to stop the risk of cancer coming back, during early stage. It is also used when chemotherapy stop responding in advanced-stage breast cancer. *(Holmes Frankie Ann.et al., 1991).*

2.1.2 VINCRISTINE

The chemotherapeutic drug vincristine is an alkaloid derived from plant thus called as plant alkaloid. It used to treat various types of cancers. The plant, *Catharanthus roseus* commonly called as The Periwinkle plant is source of vinca alkaloids. It also known to be good anti-microtubule agent as it is responsible of prevent the growth of cell's microtubule structure. Microtubules play an important role in dividing and replication of cells, so there inhibition can lead to death of cells.

This drug is given intravenously (IV) as it is not available in pill form. It has been found that vincristine acts as vesicant that is, it can cause damage to the tissue if it spills or escapes, when given from veins *(Holland, James F.et al., 1993).*

2.1.3. VINBLASTINE

Vinblastine is a plant alkaloid used as chemotherapeutic drug and a medication used for the treatment of several types of cancers such as, lung cancer, bladder cancer, Hodgkin's lymphoma, brain cancer etc. Vinblastine is given intravenously and there is no pill form available. The working action of this drug is inhibition of mitosis. The source plant for *Vinblastine* is *Catharanthus roseus*, also called *Vincarosea. (Klement Giannoula. et al.,2000).*

2.1.4 GEMCITABINE

*Gemcitabin*e is anti-metabolite anti-cancer and chemotherapeutic drug. It is used to treat the various types of cancers such as pancreatic cancer soft-tissue sarcoma, ovarian cancer bladder cancer metastatic breast cancer and non-small cell lung cancer. It's given intravenously as an infusion in veins. The dosage of gemcitabine depends upon the various factors like height and weight of patient and the type of cancer in patient. The side effect of *gemcitabine* include fever, flu like symptoms, nausea, skin rash, low blood count and poor appetite. In most people these symptoms have not been seen at all. *Gemcitabine* is anti-metabolite drug. The various substances present within the cells resemble the anti-metabolites. These anti metabolites attack at specific phase of cell cycle, when they are incorporated into cell metabolism, the cell is not able to divide. *(Burris 3rd, et al., 1997).*

In early 1980s, Larry Hertel's lab at Eli Lilly was first lab to synthesize *Gemcitabine* and was first licensed in 1995 in UK. *Gemcitabine* was first believed to be an antiviral drug but later in vitro preclinical testing revealed that it could kill leukemia cells. *(Sneaderwalter et al.,2005).*

2.1.5 TAMOXIFEN

Tamoxifen is drug used in treating patient, diagnosed with early stage, hormone-receptor-positive breast cancer. *Tamoxifen* is also being studied further by researcher for various types of cancers.

Tamoxifen was listed as very essential medication for basic health system by world health organization in their List of Essential Medicines. *Tamoxifen* is FDA approved medicine under brand name Nolvadex, available as generic medicine. It's available as pill as well as liquid form. It is not recommended for pregnant women to take this drug as it may be fetal for developing embryo. It belongs to the group triphenlethylene ("Tamoxifen Citrate"). *(Karn, A. et al.,2010).*

2.1.6. ANTI-CANCER DRUG DISCOVERY PROGRAMME

To take hold of this fetal disease cancer, it is important to continue progress in field of discovery and development from developing new therapeutic agents. National Cancer Institute (NCI) is continuing its progress in development of new drugs and has also played in important role so far. In 1995 as congress assigned an initiative called as CCNSC (Cancer Chemotherapy national service center resource to assess the potential of various anti-tumor agents that is when NCI started to develop its activities. It was in 1997 when one of division of cancer treatment and diagnosis of NCI called as DTP (developmental therapeutic program) was incorporated with functions of CCNS. The DTP since then has been busy in developing and discover various anticancer agents that are currently available in market on the large scale. *(Liscovitch, et al., 2002).*

2.2 EVALUATION OF ANTICANCER AGENTS USING FLOW CYTOMETRY ANALYSIS OF HUMAN CANCER CELLS

Flow cytometry is qualitative and quantitative laser based technology that is used to measure and analyze multiple characteristic of different cell types in heterogeneous population of cells that flow as single particle in fluid stream. It can be used to measure complexity of cytoplasm, cell size, content of DNA or RNA and can be also used to detect the membrane bound and intracellular protein. (*Michael brown. et al., 2000).*

2.2.1 PRINCIPLE OF FLOW CYTOMETRY

The flow cytometry uses monochromatic filtered light to illuminate the cells. The cells pass as single file through a narrow channel in a fluid stream. The scattering of light at various angles is detected by detector to measure internal complexities and to distinguish different cell size and the light that is emitted by cells (those labeled with fluorescent antibodies) can be used to identify cell surface antigens.

The flow cytometry is used for rapid analysis of heterogeneous cell population in precise time. The refracting light or emitting light is used by flow cytometry to identify and differentiate cells. The fluorescent dyes that are used intercalate with DNA or RNA present in the cells. After passing these cells through the light beam, excitation of these fluorescent molecules take place to high energy state. The light energy is emitted back at higher wave lengths when these fluorescent molecules return to the ground stage. The flurochromes that are used have same excitation wavelengths but different emission wave length, this property for flurochromes allows measuring various properties of cells simultaneously *(David F et al., 1994).*

Flow cytometry has been widely used in accessing apoptosis, cell division and used for isolation of various types of cells including stem cells. By using this method, we analyzed the cancer cells that were under treatment of cytotoxic plant extract to evaluate its therapeutic effect on cancer cells. The method was introduced using flow cytometry to evaluate the cytotoxicity of our plant extract, after the plant extract was applied on cancer cells. This is reliable method as well as effective method and easy to handle approach. *(Gian-jinzhanget: al., 2011)*

CHAPTER 3

METHODOLOGY

3.1 COLLECTION OF PLANT MATERIALS AND PREPARATION OF PLANT EXTRACTS

Plant material of *Petroselinum sativum a*nd *Magnolia lillifera* were taken from the local market. The leaves of plant were washed using distilled water and air dried. The soxhlet extraction method was used for the extraction overnight, following by its filtration. The process was repeated three times that will result in the concentrated sample under reduced pressure and it is then freeze dried *(Feresin et al.,2002).*

Extraction was done to separate the medicinally active portion of plant and leaving behind the insoluble cellular content or residue. The extraction could be done by using different solvents using the standard procedures. We used the solvent methanol for the extraction using soxhlet apparatus. Soxhlet extraction is also called as hot continuous extraction.

In this method of extraction, the plant material was finely chopped using piston and motor. The finely chopped plant sample was packed into the porous bag (Whatmann Paper No. 1) to prepare thimble. 60% Methanol was used as the extraction solvent. The solvent was added to plant extract and heated in the bottom flask followed by the vaporization in sample porous bag or thimble, which is then condensed in condenser and drips back. The cycle begins again after the level of solvent into the flask reaches the siphon. The process of extraction was going on for about 5 hours. After the extraction in soxhlet was complete, sample was collected and was subjected to the centrifugation at 6000 rpm. The supernatant was re-suspended into new tube while as pellet was discarded.

After this Extract was filter sterilization using filter membrane of pore size of 0.25 um, using syringe. The filtered sample was then subjected to check the cytotoxicity on cancer cell lines MCF7 and MDA-MB231 *(Luque de Castro MD. et al.,1998).*

3.2 CHEMICALS AND DRUGS

The chemicals such as DMSO, PBS, ACETIC ACID, ETHANOL etc were obtained from HiMedia, Invitogen. Propidium iodide was obtained from Sigma Aldrich, Germany. The other consumable items and raw materials were obtained from different companies such as Merck India Ltd.

3.3 CELL CULTURE AND MAINTAINENCE

Human cancer cell line MCF7 and MDAMB231 were procured from NCCS, Pune and cultured in RPMI 1640 and supplemented with 2gm/L of glutamine and 0.2% of sodium bicarbonate, 10% FBS and 1% antibiotic solution is also provided in medium. The humid atmosphere is also provided for cell growth, grown in 5% CO_2 at 37^oC. The viability of cells was assessed using different protocols, before starting the experiment. The cells that show the viability more than 95% and passage number in between 16 and 18 are used for present study.

3.4 DRUG SOLUTION

The plant extracts were dissolved in DMSO and stored in -20 ^0C. For MTT assay, 3 dilutions of the extracts were prepared viz. 10ug/mL, 30ug/mL and 50ug/mL in culture media.

3.5. MTT ASSAY

MTT assay was used to check the metabolic activity of cancer cells that were treated or untreated with extract of plant prepared earlier. The enzymes such as oxide reductase which is NAD (P)-dependent reflects the viability of cells under conditions. This enzyme produces formazan (insoluble) by reducing MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5_diphenyltetrazolium bromide], which is a tetrazolium based dye and gives purple color. Hence, MTT assay is also called as colorimetric assay.

The cells that were viable and having active metabolism converted MTT into the purple colored insoluble formazan, having absorbance maxima of about 570 nm. The dead cells were not capable of converting MTT into formazan.(*Berridge MV et al 2005*)

Protocol of MTT Assay

1. Cells were trypsinized from the mother culture and counted using trypan blue staining on a Neubauer Chamber.

2. 10,000 cells of MCF-7 and MDA-MB231 per well were seeded in 18 wells each of a 96-well plate Cells and incubated at 37^{o}C overnight.

3. Post-incubation, the cells were washed with PBS and media was changed.

4. Dilutions of extract were prepared in complete media and added to each well bearing total volume of 100 µl. The cells were then incubated at 37^{o}C for 24 hours.

5. Post treatment, the cells were treated with MTT (0.8mg/mL) and incubated for four hours at 37^{o}C.

6. The media was decanted and the wells were dried for formation of formazan crystals.

7. After this solubilization solution (DMSO) 100 µl was added to each well to dissolve formazan crystals.

8. The solubilization solution and formazan crystals were mixed properly and the absorbance was recorded at 570nm in a micro titer plate reader.

9. The data that is obtained is plotted as absorbance against number of cells which should provide the curve having linear portion. The absorbance value of cells should fall in the range of 0.75 and 1.25 and the optimal number of cells should be within the curve's linear portion. This will help in measuring the inhibition as well as stimulation of cells.

The MTT assay included:

a) Control cells that were untreated

b) Test cells that were treated with varying concentrations of *Petroselinsum sativum* plant extract

c) Wells containing medium only as blank.

3.2. PROTOCOL OF FLOW CYTOMETRY FOR THE ANALYSIS OF CELL CYCLE

3.2.1 Permeabilizing Cells, Harvesting and Fixing

a. Cells were plated at proper density to prevent confluence at the time of harvest.
b. Cells were washed with 10ml PBS and harvested by trypsinization and centrifugation (10^6cells/mL).
c. Supernatant was removed and cells were re-suspended in PBS (0.5ml).
d. Ice-cold 70% ethanol (4.5ml) was added in a drop wise manner to cell suspension while vortexing.
e. The fixed cells were incubated at -20 °C for 2hours at least prior to PI staining.
f. Cells were centrifuged at 300 rpm for 3 min and remove ethanol.
g. Cells were washed twice by centrifugation for 5mins at 200 g, with FACS buffer (5ml).
h. Supernatant was discarded and cells were re-suspended in 100 µL FACS buffer (1×10^6 cells/mL).
i. Cells were then washed with FACS buffer (5ml) twice by centrifugation.
j. Supernatant was discarded and PI staining solution was added and pellet was re-suspended.
k. Cells were incubated for 20 minutes at room temperature and analyzed with settings for Propidium Iodide in the BD FACS Verse system.

CHAPTER 4

RESULTS AND DISCUSSION

To evaluate the cytotoxic potential of plant extracts on the cancer cell line MCF-7 and MDA-MB-231, MTT assay was performed. The cytotoxicity of plant extract was determined in the dose dependent manner. The different concentrations of plant extracts showed the varying result the highest cytotoxicity was seen in highest concentration of plant extract of *Petroselinum sativum* and *Magnolia lillifera*. The cell death can be determined by morphological changes. MTT is used to check the percent cell viability. 96-well culture plates containing cells put in CO_2 incubator for 24h at 37^{o}C. After the respective exposure in CO2 incubator, MTT is added and plates are incubated for 4h. Supernatant is discarded and DMSO of 200µl concentration is added to each well and then mixed gently. Multiwall micro plate reader is used to read color development at 550 nm. (Siddiqui et al., 2008).

Name of the Plant	**Cell Line**	**Solvent**	**%cell growth Inhibition (= 100 - %proliferation)**				
			Control	5FU	10µg/ mL	30µg/ mL	50µg/ mL
Petroselinu msativum	MCF-7	60% Methanol	Proliferative	87.20%	21.30 %	45.20 %	76.60 %
	MDA-MB231		Proliferative	81.20%	23.80 %	30.90 %	81.20 %

Table 4.1: Cytotoxicity of *Petroselinum sativum* using MTT Assay

The *in-vitro* cytotoxic activity of *Petroselinum sativum* extract was estimated using MTT assay. The cells were trypsinized using 1X Trypsin-EDTA and counted using trypan blue staining. The potential biological activity of this extract was evaluated by testing the extract on human breast cancer cell lines MCF-7 and MDA-MB231. The cells were seeded in 96-well plate at a density of 10,000 cells per well. Different concentrations of plant extract were used to test the proliferation and inhibition of MCF-7 and MDA-MB231 cells. The cells were treated or untreated with 10µg/mL, 30µg/mL and 50µg/mL of respective plant extracts. 5-Fluorouracil was used as a positive control at a concentration of 5µg/mL.MCF-7 cells treated with *Petroselinum* extract, the growth inhibition was 21.30%, 45.20% and 76.60% at concentrations of 10µg/mL, 30µg/mL and 50µg/mL respectively. The percentage proliferation of the untreated cells was 117.3±5.4 for the exposure of 24 hours, which showed the cells were dividing in control. The

cytotoxic effect of methanol extract 10µg/ml, 30µg/ml, 50µg/ml showed the proliferative index of (72.7±4.2), (54.8±3.4), (23.4±2.1) respectively out of 100 cells, and the growth inhibitions index of 21.30%, 45.20%, 76.60%. Whereas the MCF-7 that were treated with 5-FU (5-fluorouracil) showed percentage proliferation of 12.8±0.8 and percentage growth inhibition of 87.20%. These readings were obtained by using spectrophotometer (table.1).

The results of the human cancer cell line MDA-MB231 inferred from the table.1.When comparing the cytotoxic effect of 5-Fluorouracil which is well known cancer cell inhibitor, with cytotoxic effect of different concentration of the *Petroselinum sativum*, the results reveal (table.1) that with the increase in extract concentration, cell inhibition also increased, this provide scope that the extract is a good candidate for further studies of activity-monitoring and identifying active anticancer principles. In a study carried out by researchers on the same cancer cell lines, (MCF-7 and MDA-MB231) aimed to check the cytotoxic potential of woody shrub *Vermonia amygdalina* (VA), result obtain from MTT assay showed that VA was inhibiting the proliferation of these cancer cell lines (MCF-7, MDA-MB231) in a dose dependent as well as time dependent manner. This drug was responsible for the cycle arrest in G1/S phase in MCF-7 cells. VA was responsible for induction of apoptosis in two cancer cell lines. This drug induced apoptosis only in the MCF-7. Our plant extract induced apoptosis in both cancer cell lines under study. VA induced apoptosis was less evident in MDA-MB231 cells. This assay was carried in both time and dose dependent manner. At highest concentration of VA, cell viability was independent of time. The study revealed that the VA was showing maximum cytotoxicity on MCF-7and MDA-MB-231 at highest dose for 48hrs. When compared to cytotoxic effect of our plant extract (in dose dependent manner) our result are very much similar to this, as the maximum inhibition of cell growth is at highest dose of plant extract for 24hrs *(Fang Cheng Wong, et al.,2013).*

In another study the plant extract of *Utrica membranacea* has showed inhibitory effect on the various human cancer cell lines which lead to apoptosis of the cells. The concentration of extract used was 3mg/ml final concentration. This plant extract was added to the 0.3×10^6/3.5 mL Hec1A cells at incubation time of 6 to 48h and viability test was also done. This plant extract could inhibit the cell growth at lower concentration. *(Elisha Solowey at el, 2014).* The extract from our

plant *Petroselinum sativum* showed less inhibitory effect on cancer cell at lower concentration whereas the inhibition rate increased with the increased concentration of extract.

The *Magnolia* extract was also evaluated for its cytotoxic potential using MTT assay. The breast cancer cell lines MCF-7 and MDA-MB231 were trypsinzed and counted using trypan blue. the cells were seeded in 96-well plate at density of 10,000 cells per well using the same protocol that was used in case of *Petroselinum sativum* extract for checking the potential biological activity of the extract. The different concentration of *Magnolia* extract was used to test the inhibition as well as proliferation of MCF-7 and MDA-MB231

Name of the Plant	**Cell Line**	**Solvent**	**%cell growth Inhibition (= 100 - %proliferation)**				
			Control	5FU	10µg/mL	30µg/mL	50µg/mL
Magnolia lilifera	MCF-7	60% Methanol	Proliferative	86.40%	35.40%	50.80%	83.20%
	MDA-MB231		Proliferative	80.40%	28.10%	44.40%	79.80%

Table 4.2: Cytotoxicity of *Magnolia lillifera* on MCF-7 and MDA-MB-231 using MTT Assay

. The percentage proliferation of untreated cells of MCF-7 was 128.4±6.1 and that of MDA-MB231 was 146±8.2 for the exposure of 24hours that showed cells were dividing in control. The cytotoxic effect of methanolic extract of *Magnolia lillifera* of concentration 10mg/ml, 30mg/ml, 50mg/ml showed the proliferative index of 64.6±6.3%, 49.2±2.9%, 16.8±0.6% respectively (Fig. 4.1) and the growth inhibition of 35.40%, 50.80%, and 83.20% on the cell line MCF-7. The MCF-7 that was treated with 5-FU (5-fluorouracil) showed the percentage proliferation of 13.6±1.1 and percentage growth inhibition was 86.40%.

In case of human breast cancer cell line MDA-MB231 proliferative index for concentration of *Magnolia* extract 10mg/ml, 30mg/ml, 50mg/ml was found to be (19.6±2.1), (71.9±5.1), (55.6±3.9) respectively (Fig. 4.2), and the growth inhibitions index of 28.10%, 44.40% and 79.80% respectively for the above concentrations of extract. While as MDA-MB231 that was treated with anticancer drug 5-Fluorouracil showed the percentage proliferation of 19.6±2.1 and percentage growth inhibition was 80.40%.

When the cytotoxicity of 5-flouro uracil, which is well known anti-cancer drug, is compared with cytotoxicity potential of different concentration of methanolic extract of *Magnolia*, it can be seen from the (table 2) that with the increase in extract concentration, cell inhibition also increased, thus it can be concluded that this extract is good candidate for active monitoring and identifying active anti-cancer principles.

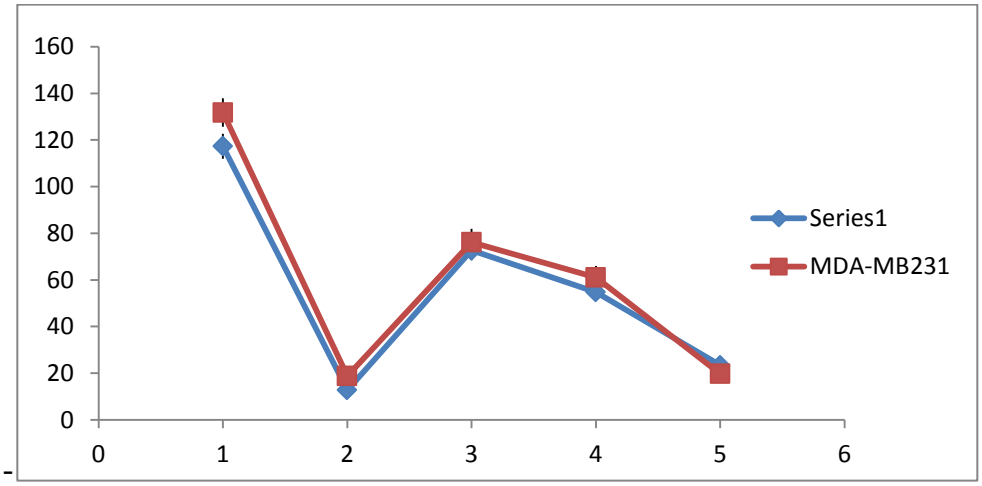


Figure 4.1: **Cytotoxicity of petroselinum extracts on MCF-7 and MDA-MB231. (Series1- MCF-7)**

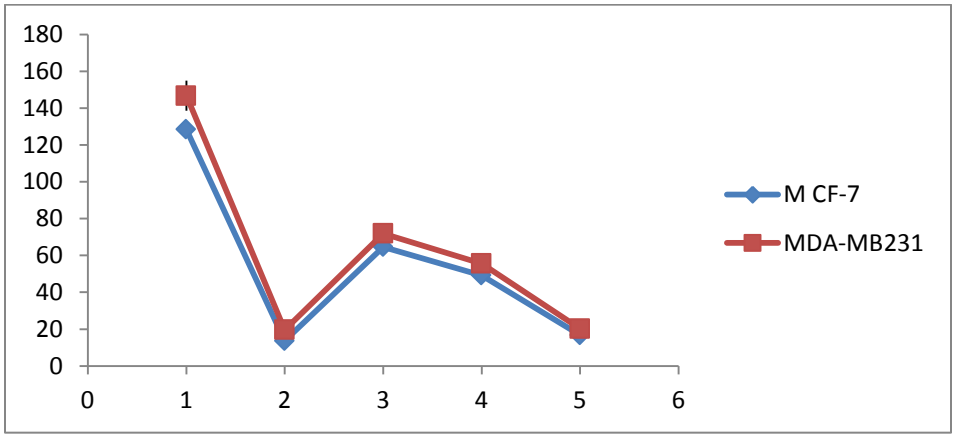


Figure 4.2: Cytotoxicity of *Magnolia* extracts on MCF-7 and MDA-MB231

In another phytochemical study of plant *Andrographis paniculata*, 14 compounds were isolated from the aerial parts of this plant and their ethanol extract were added to the cancer cell line HepG2 and colon 205 tumor cells. The bioactivity assay revealed that these metabolites showed the cytotoxic activity against above mentioned human cancer cell lines. Therefore, the plant

derived compounds can be effective in inhibition of various cancer cell lines. *(Geethangili M et al.,2008).*

4.1 Cell Cycle Analysis Using Flow Cytometry

The cell cycle distribution and cell proliferation assessment is important to study the cell differentiation and apoptosis. Assessing these properties of cells will help in investigation of basic underlying mechanism and will also enable to evaluate the efficiency of anti-cancer drug and its therapeutic potential. The cell cycle under goes a progression of G1→S→G2→M in proliferating cells for the synthesis of DNA where as in case of G0 phase, cell neither proliferates nor divides. The cells that enter G0 phase are having minimal cellular function and less cell cycle machinery. This stage of cells is also called as quiescent stage. *(Kim, Kang Ho. et al., 2015).*

The analyses of cell cycle can be done by quantification of DNA content in the cell. The cells are stained using various dyes that bind to the DNA. These dyes have the property of binding to proportion of DNA that is present in the cell. The cells in S phase have more DNA than the cells that are in G1 phase of cell cycle, which means the cells in S phase will take up more dye proportionally and will fluoresce more. After the doubling of DNA content, the cells in G2 phase will be twice bright as cells that are in G1 phase. *(Kang ho kim et al.,2015)*

The most commonly used DNA-binding dye is Propidium iodide (PI). Other dyes that are being used are 7-aminoactinomycin-D (7-ADD), DRAQ7™and DRAQ5™, Hoechst 33342, 33258 and S769131 etc.

To identify resting stage and proliferating cell fraction, there are certain proliferation-associated proteins and nuclear proliferation antigen that are identified such as Ki-67 and cell nuclear antigen for proliferation (PCNA). In the G0 or resting stage of cell cycle Ki-67 is rarely detected and in G2 and early M phase, its expression is maximum. Also, PCNA in proliferating cells is good marker and appears concentrated in S phase.

As the study suggests that RNA content is usually high in the proliferating cells as compared to the resting cells, so RNA quantification can be alternative method for studying the cell cycle

status. Pyronin Y is commonly used dye to observe the intracellular RNA in combination with the other dye called as methyl green. *(Kang ho Kim et al.,2015).*

4.2Flow Cytometry Result on Cell Line Mcf-7 Treated With *Magnolia* Extract

MCF-7, treated with *Magnolia* plant extract, show increased cytotoxic result in MTT assay. To determine the mechanism of cell proliferation inhibition by *Magnolia* extract, MCF-7 (treated with magnolia extract) was subjected to flow cytometry for cell cycle analysis.

The proliferative cells were at the different stage of cell cycle, so before the analysis of cells MCF-7 cells were synchronized by treating with 10µg/mL Nocodazole for 48 hours. After the exposure of 48hrs, the cells were placed in complete media to resume cell cycle along with 50µg/mL of *Magnolia* extract. The treatment was given for 24 hours. Next, the cells were trypsinized and collected by centrifugation at 150xg. The cell pellet was washed thoroughly with 70% ice-cold ethanol for the permeabilization and stained with Propidium iodide.

4.3 Cell Cycle Analysis

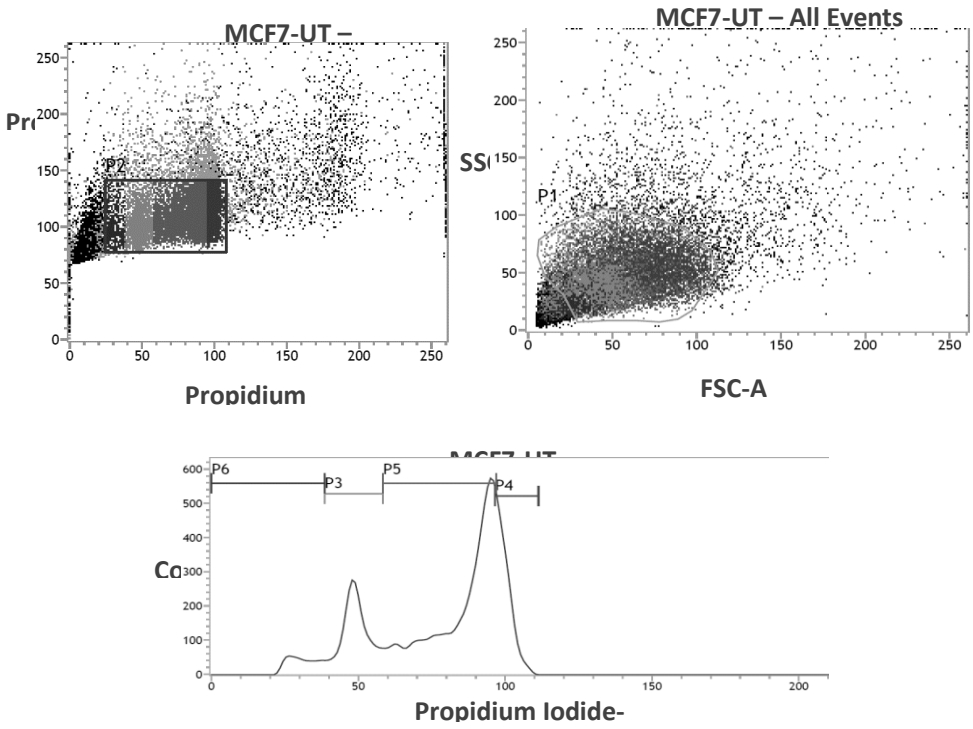


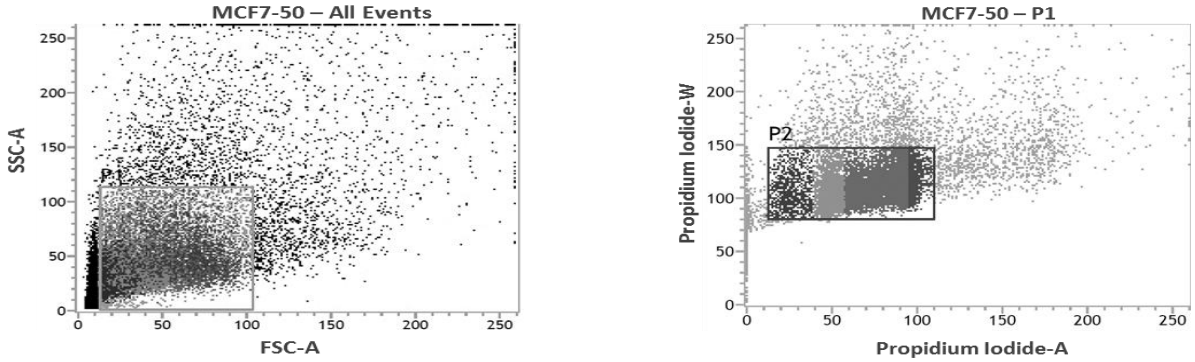
Figure 4.3: Cell Cycle analysis of untreated MCF-7 cells using Flow Cytometry

Name	Events	%Parent	%Grandparent	%Total	Propidium Iodide-A Mean	Propidium Iodide-A CV
MCF7-UT:P3	2947	**22.32**	19.01	14.73	49,854	7.01
MCF7-UT:P4	4294	**29.95**	26.25	21.47	98,965	3.05
MCF7-UT:P5	6610	**52.65**	44.09	33.05	82,668	16.51
MCF7-UT:P6	1023	**7.75**	6.60	5.11	28,659	27.21

Table 4.3: Flow Cytometry results of untreated MCF-7

The populations P3 represents G1 phase whereas P4 represents G2 Phase. The cell cycle analysis in human breast cancer cell line MCF-7 was performed using flow cytometry. Experiment was performed in triplicates. Propidium iodide was used for cell staining. The above fluorescence histogram (Fig. 4.3) shows the cell cycle distribution of untreated MCF-7 cells (UT). The histogram generated from the scattered graph shows that the most of cells (P4) are at G2, as the highest peak is obtained between 150-100 on the histogram of untreated cells. This amply those cells are at the dividing phase and are undergoing replication.

4.4CELLS TREATED WITH *MAGNOLIA* EXTRACT



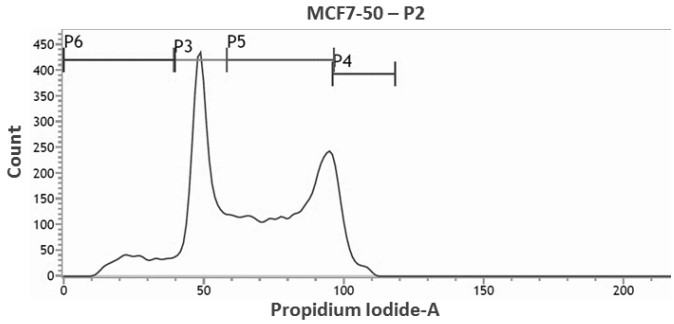


Figure 4.4: Cell Cycle analysis of MCF-7(treated with *Magnolia* extract) using flow cytometry.

Name	Events	%Parent	%Grandparent	%Total	Propidium Iodide-A Mean	Propidium Iodide-A CV
MCF7-50:P3	3843	**36.60**	30.02	19.21	50,094	7.34
MCF7-50:P4	1325	**12.61**	10.35	6.62	100,121	3.19
MCF7-50:P5	5464	**52.03**	42.68	27.32	79,850	14.89
MCF7-50:P6	942	**8.97**	7.35	4.71	26,897	25.45

Table 4.4: Flow Cytometric results of MFC-7 treated cells with *Magnolia* Extract

The histogram obtained from the scattered plot of treated cells (with *Magnolia* extract) shows the shift of from G2 Phase to G1 phase of cell cycle, as the highest peak is obtained between 100-50 (arbitrary number corresponding to the DNA content at different phases of cell cycle) on histogram of treat cells of MCF-7. This indicates that G2 phase arrest has occurred, cells are not dividing. It can be concluded that shift of cell cycle from G2 phase to G1 phase of cell indicate the cell death by apoptosis.

CHAPTER 5

CONCLUSION

The methanol extract from the *Petroselinum sativum* and *Magnolia lillifera* exhibited the cytotoxic capability on MCF-7 and MDA-MB231 cancer cell line. The plant extracts showed the increased inhibitory effect in a dose-dependent manner. The cytotoxicity of both plant extract was highest at the concentration of 50µg/mL for a period of 24 hours of treatment. The treatment was found to be more effective in MDA-MB231 cell line at the higher concentration of plant extract in case of *Petroselinum sativum*. The plant extract from *Magnolia* show highest cytotoxicity in MCF-7. As per the data from mechanistic studies of flow cytometry, *Magnolia* extract caused cell cycle arrest in G1 Phase, which is most likely to lead the cell to apoptosis. This provides great scope for these plants in medicinal applications. Results suggested that the extracts at different concentration showed promising anticancer activity.

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